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Critical Review

Firefly Bioluminescence: A Mechanistic Approach of Luciferase Catalyzed Reactions

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Summary

Luciferase is a general term for enzymes catalyzing visible light emission by living organisms (bioluminescence). The studies carried out with *Photinus pyralis* (firefly) luciferase allowed the discovery of the reaction leading to light production. It can be regarded as a two-step process: the first corresponds to the reaction of luciferase's substrate, luciferin (LH_2), with ATP-Mg^{2+} generating inorganic pyrophosphate and an intermediate luciferyl-adenylate (LH_2-AMP); the second is the oxidation and decarboxylation of LH_2-AMP to oxyluciferin, the light emitter, producing CO_2, AMP, and photons of yellow-green light (550–570 nm). In a dark reaction LH_2-AMP is oxidized to dehydroluciferyl-adenylate (L-AMP). Luciferase also shows acyl-coenzyme A synthetase activity, which leads to the formation of dehydroluciferyl-coenzyme A (L-CoA), luciferyl-coenzyme A (LH_2-CoA), and fatty acyl-CoAs. Moreover luciferase catalyzes the synthesis of dinucleoside polyphosphates from nucleosides with at least a 3′-phosphate chain plus an intact terminal pyrophosphate moiety. The LH_2 stereospecificity is a particular feature of the bioluminescent reaction where each isomer, D-LH_2 or L-LH_2, has a specific function. Practical applications of the luciferase system, either in its native form or with engineered proteins, encloses the analytical assay of metabolites like ATP and molecular biology studies with *luc* as a reporter gene, including the most recent and increasing field of bioimaging.

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Keywords bioluminescence; luciferase; acyl-CoA synthetase; L-luciferin; D-luciferin; oxyluciferin; dehydroluciferyl-adenylate.

Abbreviations AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CoA, coenzyme A; L, dehydroluciferin; L-AMP, dehydroluciferyl-adenylate; L-CoA, dehydroluciferyl-coenzyme A; LH_2, firefly luciferin; LH_2-AMP, luciferyl-adenylate; LH_2-CoA, luciferyl-coenzyme A; NTP, nucleoside 5′-triphosphate; PPi, inorganic pyrophosphate.

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INTRODUCTION

Bioluminescence, the result of a process that occurs in living organisms in which an electronically excited substance produced in a chemical reaction decays to the ground level (*1*), is a widespread phenomenon in Nature. The scientific research on this subject led to the discovery of the enzyme-substrate system, the former called luciferase and the latter luciferin (from the Latin *Lucifer*, "Light-bringer"). Among all the bioluminescent organisms fireflies are the most studied and well-characterized, specially the North American firefly, *Photinus pyralis* (Order Coleoptera, Family Lampyridae).

The purpose of this review is to present the chemical reactions catalyzed by *Photinus pyralis* luciferase, furnishing basic information about its principal components, luciferase and luciferin. Also, some recent applications of the enzyme will be summarized together with well-established methods.

FIREFLY LUCIFERASE

Firefly luciferase is classified as *Photinus*-luciferin: oxygen 4-oxidoreductase (decarboxylating, ATP-hydrolysing) (EC 1.13. 12.7). Its substrate is firefly luciferin (LH_2), and the reaction requires ATP, oxygen and a metallic cation (*2, 3*). Crystallographic studies have shown that the protein is folded into two compact domains, a large N-terminal domain and a small C-terminal domain, joined by a flexible linker peptide, which creates a wide cleft between the two domains [Fig. 1; (*4*)]. The C-terminal portion bears the tripeptide Serine-Lysine-Leucine (SLK motif) responsible for peroxisome targeting (*4, 5*). The putative active site is believed to enclose amino acid residues on the surface of both domains, which suggests that during the course of the reaction the two domains will come together and cluster the

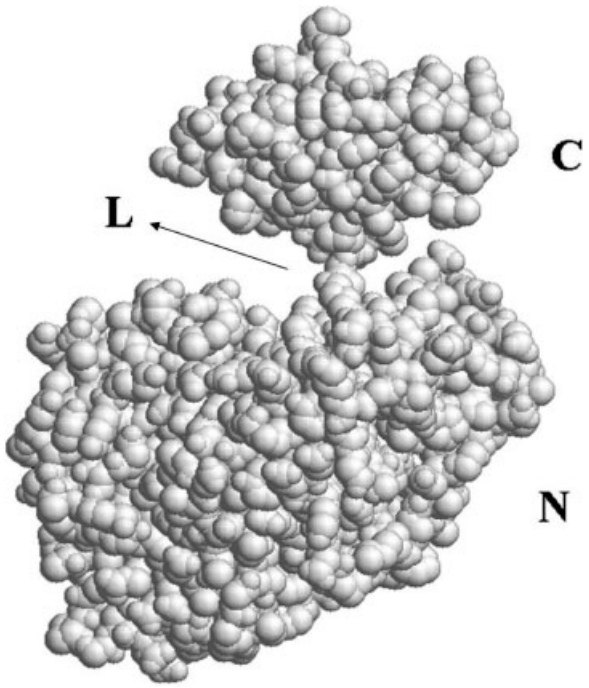

Figure 1. *Photinus pyralis* luciferase (Protein Data Bank accession number 1LCI) without its substrate in spacefill display. The image depicts the large N-terminal domain (N) and the small C-terminal domain (C), linked by a short loop (L). In this side-view representation the cleft between the two domains is visible. The image was generated using the program Rasmol version 2.7.3 (Raswin Molecular Graphics©).

substrate between them, requiring a significant conformational change (*4*). Indeed, studies with wild-type and red mutant luciferase from *Luciola cruciata* showed that native luciferase adopts a "closed form" during the formation of the high-energy intermediate responsible for light emission, creating a hydrophobic pocket around the active site, and an "open form" in complex with the reactants and products (*6*). The major part of the amino acid residues important to the bioluminescent activity is located in the N-terminal portion and only one in the C-terminal domain (*7*), and it was demonstrated that luciferase can produce light bearing only the N-domain, albeit with a luminescent output of only 0.03% of the complete enzyme (*8*).

The typical emission spectrum for luciferase is in the yellow-green region (550–570 nm), with a peak at 562 nm at basic media (pH $\sim$ 7.5–7.8) (*9*). However, luciferase is a pH-sensitive enzyme, and acid media (pH $\sim$ 5–6) can shift the emission to red (maximum at 620 nm), as well as higher temperatures and heavy metal cations (*10*). It is believed that conformational changes, which influence the active site microenvironment, are responsible for the different color emission (*6*). In *Luciola cruciata* red mutant luciferase the catalytic state remains in an "open form," which could allow energy loss from the excited state intermediate, leading to the emission of red light instead of the higher-energy yellow-green light (*6*).

The *in vitro* emission of light follows, under well-defined conditions, a flash pattern, with a rise in the intensity of emission that decays to low levels (about 5% of the initial burst) in a few seconds, even in the presence of available substrate [Fig. 2; (*3*)]. This profile was attributed to the formation of inhibitory products in the course of the reaction (*11, 12*), a topic to be discussed later.

Firefly luciferin (LH_2) has the chemical formula [(*S*)-2-(6′-hydroxy-2′-benzothiazolyl)-2-thiazoline-4-carboxylic acid] [Fig. 3; (13–15)], which was proposed and confirmed on the basis of chemical synthesis (*13*) and X-ray crystallography (*16*). In Nature, however, the biochemical pathway for LH_2 synthesis remains a mystery (*15*). In fact it is not even known if the fireflies contain all enzymes to synthesize LH_2 or if it is obtained by ingestion of other bioluminescent organisms or by symbiosis with bacteria (*15*). Also, the evolutionary creation of LH_2 is questioned. Some researchers propose that LH_2 first served as an antioxidant, later being requested by the bioluminescent reaction when luciferase appeared (*17*). It has been suggested that the origin of the thiazoline ring could be a cysteine (*15*), as can be observed in the chemical synthesis in which one of the steps involves this amino acid (*18*). Cysteine has two isomers, L- and D-, and either one of them can be used to form LH_2, giving rise to L-LH_2 and D-LH_2, respectively. Their function in the bioluminescent reaction will be highlighted in the incoming sections.

FIREFLY LUCIFERASE REACTIONS

The bioluminescent reaction starts with the reaction of D-LH_2 with ATP, as expressed in Eq. (1) [Fig. 4; (*19–22*)].

$$\text{Luciferase} + \text{D-LH}_2 + \text{ATP-Mg}^{2+} \rightleftharpoons \text{Luciferase} \cdot \text{D-LH}_2\text{-AMP} + \text{PPi-Mg}^{2+} \quad (1)$$

The reaction is a S_N2 nucleophilic displacement, which involves the carboxylic group at the C_4 carbon at the thiazoline ring of D-LH_2 and the phosphate groups of ATP. The oxygen in

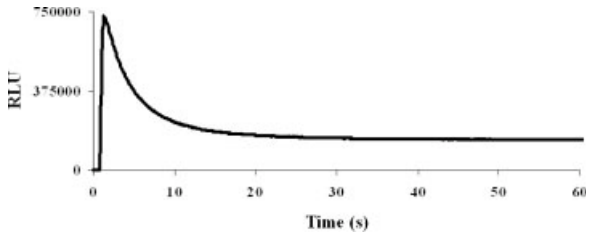

Figure 2. *In vitro* profile of light production by luciferase with high substrate concentrations (D-LH_2 and ATP-Mg^{2+} in the μM range). After a rapid increase in the light output to a maximum an accentuated decay in the intensity of emission is verified. (RLU, relative light unit).

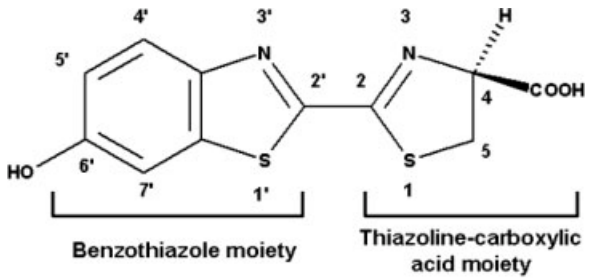

Figure 3. Chemical structure of firefly luciferin, D-LH_2. The two principal moieties of the molecule are shown, with the atoms numbering. Figure adapted from ref. 15.

the carboxyl group is a nucleophile, and nucleophilic attack of the electrophilic phosphorus at the α-phosphoryl group of ATP displaces inorganic pyrophosphate (PPi), an excellent leaving group, and transfers adenylate (5′-AMP) to D-LH_2. The reaction is, thus, an adenylylation, and produces an enzyme-bound intermediate, luciferyl-adenylate (LH_2-AMP) (*22–24*), a mixed anhydride. The phosphoryl group transfer is also found, for example, in the activation of fatty acids by fatty acyl-coenzyme A synthetases and in the attachment of amino acids to their correspondent tRNA in protein synthesis by aminoacyl-tRNA synthetases (*21, 25*). Formation of ATP-Mg^{2+} complexes partially shields the negative charges and influences the conformation of the phosphate groups, and thus explains the requirement of this divalent cation in the reaction (*26*).

The L-LH_2 isomer can also be adenylylated, giving rise to L-LH_2-AMP; however it is not used further in light production (*24, 27*).

The attachment of a good leaving group like AMP to a metabolic intermediate activates it for subsequent reaction, in this case the oxidation and decarboxylation of the formed D-LH_2-AMP [Eq. (2)] [Figs. 5 and 6; (*21, 28–30*)].

$$\text{Luciferase} \cdot \text{D-}LH_2\text{-AMP} + O_2 \rightarrow \text{Luciferase} + \text{AMP} + CO_2 + \text{oxyluciferin} + \text{photon} \quad (2)$$

After the formation of D-LH_2-AMP the C_4 carbon looses a proton, creating a carbanion. This mechanism explains the function of the adenylylation step: ATP would not serve as an energy donor (*23*) but instead increases the acidity of the C_4 carbon (*30, 31*). The carbanion undergoes a nucleophilic attack of molecular oxygen, resulting in a linear hydroperoxide (*30, 32*). The displacement of AMP favors the intramolecular nucleophilic attack of the α-hydroperoxy group towards the formation of a strained four-membered cyclic intermediate, the luciferin dioxetanone (*33*), an energy-rich moiety that spontaneously breaks up generating CO_2. Double-labelling experiments with $^{18}O_2$ and $H_2{}^{18}O$ revealed the origin of the oxygen atoms in CO_2: in a $H_2{}^{16}O$ medium with $^{18}O_2$ the major portion of the CO_2 produced (up to 75%) contained one atom of ^{18}O, which suggests that one of the oxygen of CO_2 arises from the O_2 that oxidizes LH_2, and not from the solvent, thus supporting the dioxetanone mechanism [Fig. 6; (*34*)]. The resulting molecule, oxyluciferin, is in a singlet excited state, and its decay to the ground state releases a photon (*35–37*).

Besides the light-producing pathway luciferase catalyzed bioluminescence also displays lateral reactions. In such reaction the complex luciferase·D-LH_2-AMP reacts with oxygen in a dark reaction pathway leading to the oxidized product dehydroluciferyl-AMP (L-AMP) [Eq. (3)] (*23, 38–40*).

$$\text{Luciferase} \cdot \text{D-}LH_2\text{-AMP} + O_2 \rightleftharpoons \text{Luciferase} \cdot \text{L-AMP} + H_2O_2 \quad (3)$$

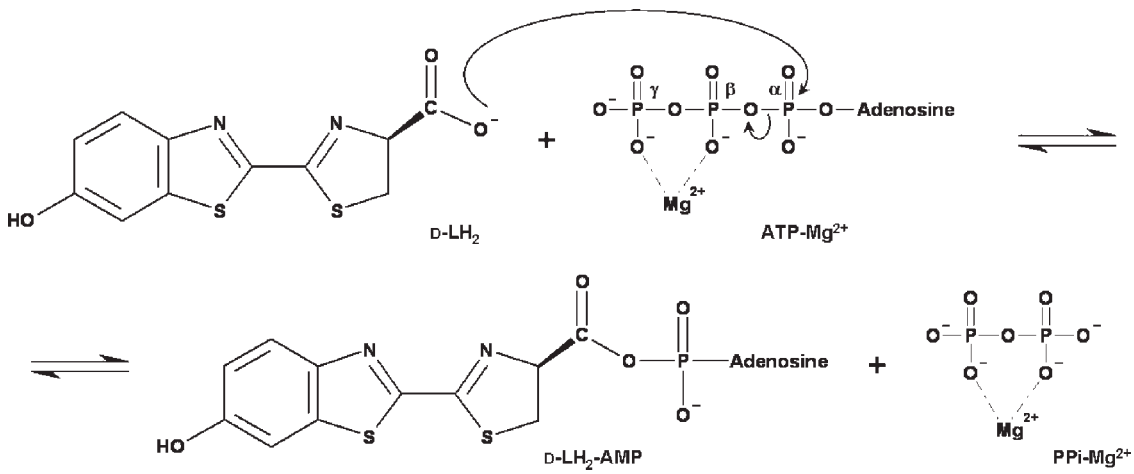

Figure 4. Chemical mechanism of the adenylylation of D-LH_2. The reaction involves the displacement of inorganic pyrophosphate (PPi) as a leaving group from the ATP molecule, which leads to the formation of the intermediate D-luciferyl-adenylate (D-LH_2-AMP).

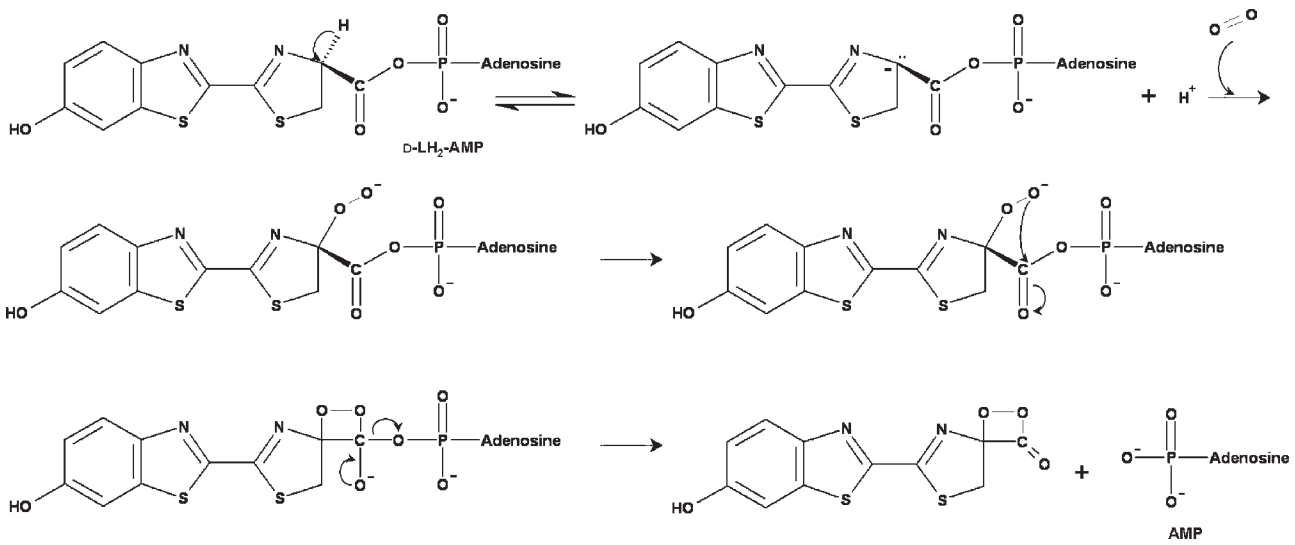

Figure 5. Mechanism of dioxetanone formation from D-LH_2-AMP. After its formation the intermediate D-LH_2-AMP looses a proton to generate a reactive carbanion, whose nucleophilic attack of molecular oxygen creates a hydroperoxide. Internal nucleophilic attack of the hydroperoxide to the electrophilic carbon of the carbonyl group displaces AMP as a leaving group and produces the cyclic dioxetatone ring, an energy-rich moiety.

If the formation of hydrogen peroxide was a natural consequence of the oxidative process *in vitro*, and that was in fact already demonstrated (*41*), such metabolite could impair biological functions if its concentration is different from the cellular demand. However the existence of luciferase in fireflies' peroxisomes (*5*), an organelle rich in the oxygen detoxifying enzyme catalase, could represent a proof that this reaction is not impossible *in vivo* (*41*).

By its turn L-AMP is capable of reacting with the PPi-Mg^{2+} released in the light-producing pathway forming dehydroluciferin (L) and regenerating ATP [Eq. (4)] [Fig. 7; (*23, 38*)].

$$\text{Luciferase} \cdot \text{L-AMP} + \text{PPi-Mg}^{2+} \rightleftharpoons \text{Luciferase} + \text{ATP-Mg}^{2+} + \text{L} \qquad (4)$$

As L is an oxidative product of LH_2 and ATP, through the oxidation of LH_2-AMP to L-AMP and its posterior pyrophosphorolysis (*23, 38–40*), it was originally regarded as the light emitter and equivocally called oxyluciferin (*19, 20, 23, 38*). Its chemical synthesis was achieved (*18*) and its role on the bioluminescent reaction, as well as of its adenylated analog L-AMP, has been clarified. The incubation of chemically synthesized L

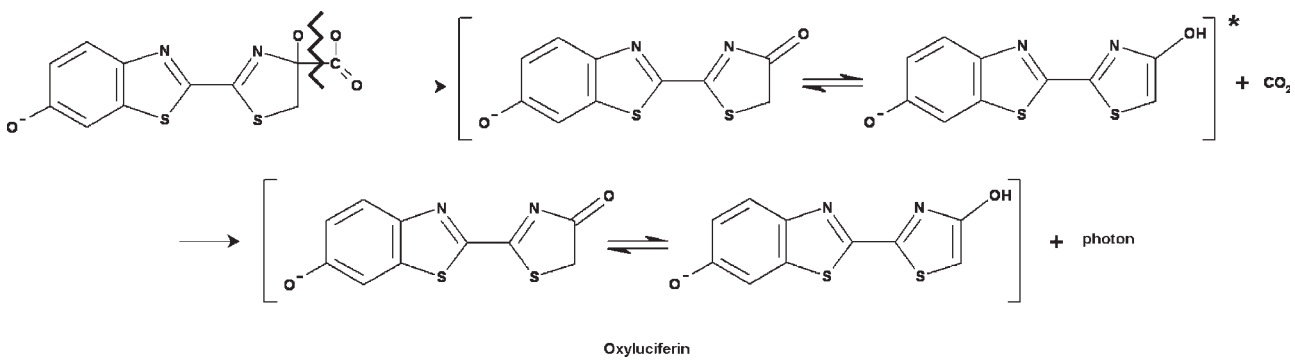

Figure 6. Oxidative decarboxylation of the dioxetanone ring in D-LH_2 to yield the light-emitter oxyluciferin. The spontaneous break-up of the dioxetanone ring, through a process not yet fully understood, produces oxyluciferin in a singlet excited state (represented by the *) whose decay to the ground level results in the emission of a photon of visible light. The keto and enol tautomers of oxyluciferin are represented in square brackets.

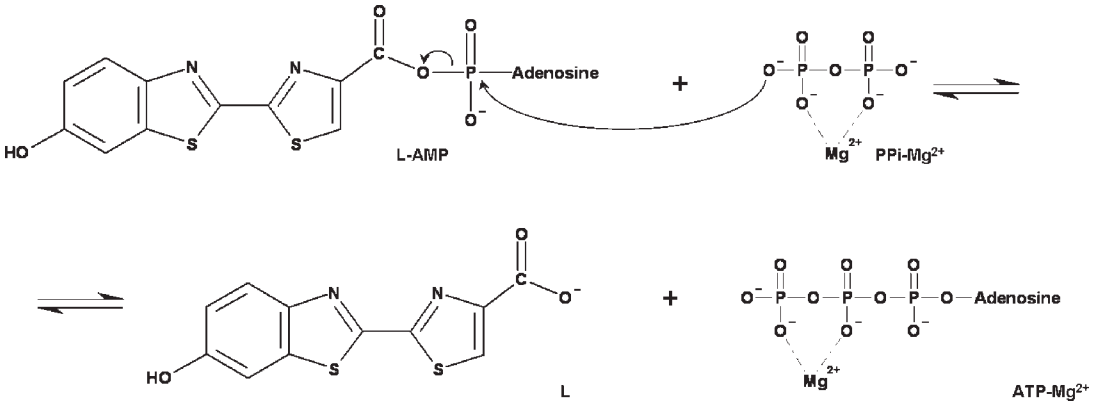

Figure 7. Pyrophosphorolysis of dehydroluciferyl-adenylate (L-AMP). In this reaction the nucleophilic oxygen at PPi attacks the electrophilic phosphorus at the adenylate moiety of L-AMP, displacing ATP and creating dehydroluciferin (L).

with luciferase and ATP-Mg^{2+} prior to the addition of LH_2 led to inhibition of the light production (*39*). This effect could be exerted not directly by L but instead through its adenylylation to L-AMP. L-AMP is a very strong luciferase inhibitor (*20, 23, 39, 42, 43*) and its synthesis from LH_2-AMP can account for about 20% of the LH_2 consumed (*40, 41*).

Besides L-AMP the other product of the luciferase-catalyzed reactions that shows marked inhibitory effect is oxyluciferin. These two products are the main responsible for the typical flash pattern of the bioluminescent emitted light. Oxyluciferin is a competitive inhibitor of luciferase ($K_i = 0.50 \pm 0.03$ μM) while L-AMP act as a tight-binding competitive inhibitor ($K_i = 3.8 \pm 0.7$ nM) (*44*).

The formation of L from L-AMP and PPi, although being a lateral reaction, brings benefits to the light-production pathway: a strong inhibitor, L-AMP, is substituted for a less powerful inhibitor, L, which can be more easily removed from luciferase's active site, thus liberating the enzyme for another cycle of reaction (*23, 42, 45*). It is also curious to verify that PPi can be simultaneously an inhibitor of the light reaction (as a product of reaction) and an enhancer at low concentrations (as it removes the strong inhibitor L-AMP through its pyrophosphorolysis to L and ATP) (*11, 42*).

Luciferase as an Acyl-CoA Ligase: Roles of CoA and NTP

The influence of coenzyme A (CoA) in luciferase light-emitting reaction is a well-established phenomenon (*38*): when added to the reaction mixture in the beginning it could stabilize the light emitted, avoiding the characteristic flash profile. Also, when added to a mixture that already produced light, it could induce a secondary flash of light (*38*). But its role on the bioluminescent reaction was not well understood since CoA is not an original reagent in the light reaction. It was demonstrated that CoA is in fact a substrate, reacting with L-AMP and producing dehydroluciferyl-CoA (L-CoA) in a luciferase catalyzed reaction [Eq. (5)] (*38, 39, 43*).

$$\text{Luciferase} \cdot \text{L-AMP} + \text{CoA} \rightleftharpoons \text{Luciferase} + \text{AMP} + \text{L-CoA} \quad (5)$$

In this scheme the role of CoA could be better explained: by reacting with the strong inhibitor L-AMP another product is formed, L-CoA. This new product is a less powerful inhibitor, so luciferase can continue catalyzing the bioluminescent reaction (*43, 45*).

The chemical synthesis of L-CoA, from L and CoA in the presence of carbonyldiimidazole bypassing the adenylylation step of L to L-AMP, allowed its chemical characterization and confirmed its identity as a product of luciferase catalyzed thiolysis of L-AMP (*45*). Also, it was demonstrated the reversibility of the reaction, through the formation of L-AMP from L-CoA and AMP and posterior pyrophosphorolysis of L-AMP to give L (*45*).

Besides L-AMP, CoA also reacts with L-LH_2-AMP (*24, 46*), leading to L-luciferyl-coenzyme A (L-LH_2-CoA) [Eq. (6)]. As L-LH_2, and hence its adenylated form L-LH_2-AMP, functions as an inhibitor of light production (*46, 47*), its reaction with CoA promotes the light-producing pathway.

$$\text{Luciferase} \cdot \text{L-LH}_2\text{-AMP} + \text{CoA} \rightleftharpoons \text{Luciferase} + \text{AMP} + \text{L-LH}_2\text{-CoA} \quad (6)$$

But how luciferase, a monooxygenase, could react with CoA? A first highlight to this question came from the observation of the resemblance of the biochemistry of the reactions catalyzed by luciferase and some ligases, like fatty acyl-CoA synthetases (*25*). These enzymes catalyze the adenylylation of fatty acids thought its carboxylic acid moiety, with release of PPi, and posterior thioesterification with CoA, in a mechanism very similar to the adenylylation step of luciferase bioluminescent reaction [Eqs. (7) and (8)] (Fig. 8).

$$\text{Fatty acid} + \text{ATP-Mg}^{2+} \rightleftharpoons \text{fatty acyl-AMP} + \text{PPi-Mg}^{2+} \quad (7)$$

$$\text{Fatty acyl-AMP} + \text{CoA} \rightleftharpoons \text{fatty acyl-CoA} + \text{AMP} \quad (8)$$

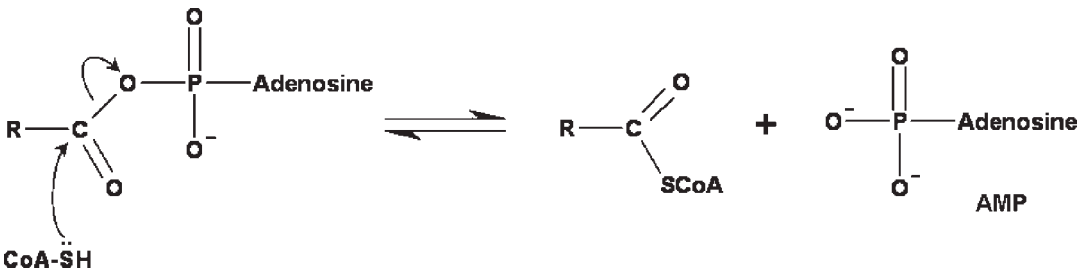

Figure 8. Mechanism of acyl-adenylate formation. In this reaction, catalyzed by synthetases and luciferase, after the adenylylation of the substrate the carbonyl carbon is attacked by the nucleophilic sulphur in the thiol group of coenzyme A (CoA), displacing AMP and creating an acyl-CoA intermediate. R denotes any other part of the substrate not involved in the reaction described.

After the production of an adenylated intermediate the thiol group of CoA carries out a nucleophilic attack on the mixed anhydride, displacing AMP and forming the thioester acyl-CoA. The question if luciferase is really a descendent of a primitive fatty acyl-CoA synthetase still remains open to discussion, but it was demonstrated that luciferase, in the presence of fatty acids, ATP-Mg^{2+} and CoA, can catalyze the formation of fatty acyl-CoAs (*48*). The structural similarities between LH_2 and arachidonic acid, one of the fatty acid tested as substrate (*48*), could account for luciferase reconnaissance and reaction [Fig. 9; (*15*)]. As fatty acids behave as inhibitors of luciferase reaction (*49*) their conversion to fatty acyl-adenylates represents another way of favoring the light production.

Besides the demonstration of the ligase-like activity of luciferase, the inverse reaction was also verified: the luciferase-like activity of ligases (*50*). In this study, using larval *Tenebrio molitor*, a non-luminescent beetle distantly related to fireflies, it was shown that the larvae shines red when injected with LH_2, with kinetics parameters similar to those of firefly luciferase, and its fat body extracts produce luminescence *in vitro* (*50*). However, in a recent study in which these luciferase-like ligase genes were cloned and produced in a functional form, the above results were not confirmed: no light was produced *in vitro* by the addition of D-LH_2 and ATP-Mg^{2+} (*51*).

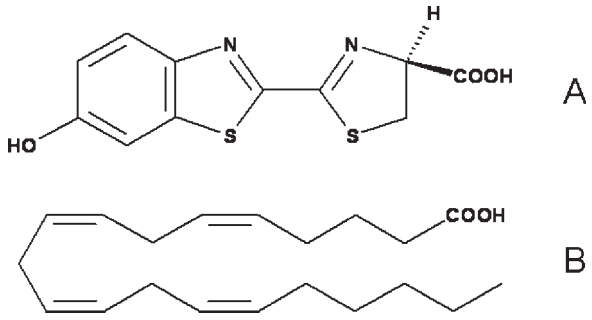

Figure 9. Comparison of the chemical structures of (A) firefly D-LH_2 and (B) arachidonic acid, a fatty acid. In (B) arachidonic acid is in its preferred (most stable) conformation, thus highlighting the spatial resemblance between it and D-LH_2. Figure adapted from ref. 15.

Another consequence of luciferase's function as a ligase is the synthesis of dinucleoside polyphosphates, in a reaction analogous to the pyrophosphorolysis of L-AMP to give ATP [Fig. 7; (*40, 52*)]. The synthesis of these "mysterious" compounds, found in numerous organisms but without a clear biological function (*53*), was demonstrated to be catalyzed by luciferase, like does acyl-CoA synthetase from *Pseudomonas fragi* (*54*), from L-AMP and any substance with an intact terminal phosphate, like nucleoside 5′-triphosphates (NTPs, for example CTP, UTP and ATP) and tripolyphosphate (P_3), as acceptors of the AMP moiety [Eqs (9)–(11)] (*40, 42, 55*), giving adenosine (5′)tetraphospho(5′)nucleoside (Ap_4N) or adenosine 5′-tetraphosphate (p_4A) as products, respectively. The formation of adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) from ATP could account for the consumption of the ATP diverged from the bioluminescent reaction. Nonetheless the best conditions for the synthesis of dinucleoside polyphosphates are the opposite of those necessary for light production, namely acidic medium, while luciferase requires a physiological pH, about 7.5, to produce light (*55*). This contradictory effect was explained as the low pH could facilitate the transfer of the adenylate moiety of L-AMP to the acceptor (*55*).

$$\text{Luciferase} \cdot \text{L-AMP} + \text{ATP-Mg}^{2+} \rightleftharpoons \text{Luciferase} + \text{L} + \text{Ap}_4\text{A-Mg}^{2+} \quad (9)$$

$$\text{Luciferase} \cdot \text{L-AMP} + \text{NTP-Mg}^{2+} \rightleftharpoons \text{Luciferase} + \text{L} + \text{Ap}_4\text{N-Mg}^{2+} \quad (10)$$

$$\text{Luciferase} \cdot \text{L-AMP} + \text{P}_3\text{-Mg}^{2+} \rightleftharpoons \text{Luciferase} + \text{L} + \text{p}_4\text{A-Mg}^{2+} \quad (11)$$

Once again L is produced, explaining the slightly activator effect verified for the referred acceptors (*39, 42*).

Figure 10 resumes the reactions enumerated above and shows the interconnections between them.

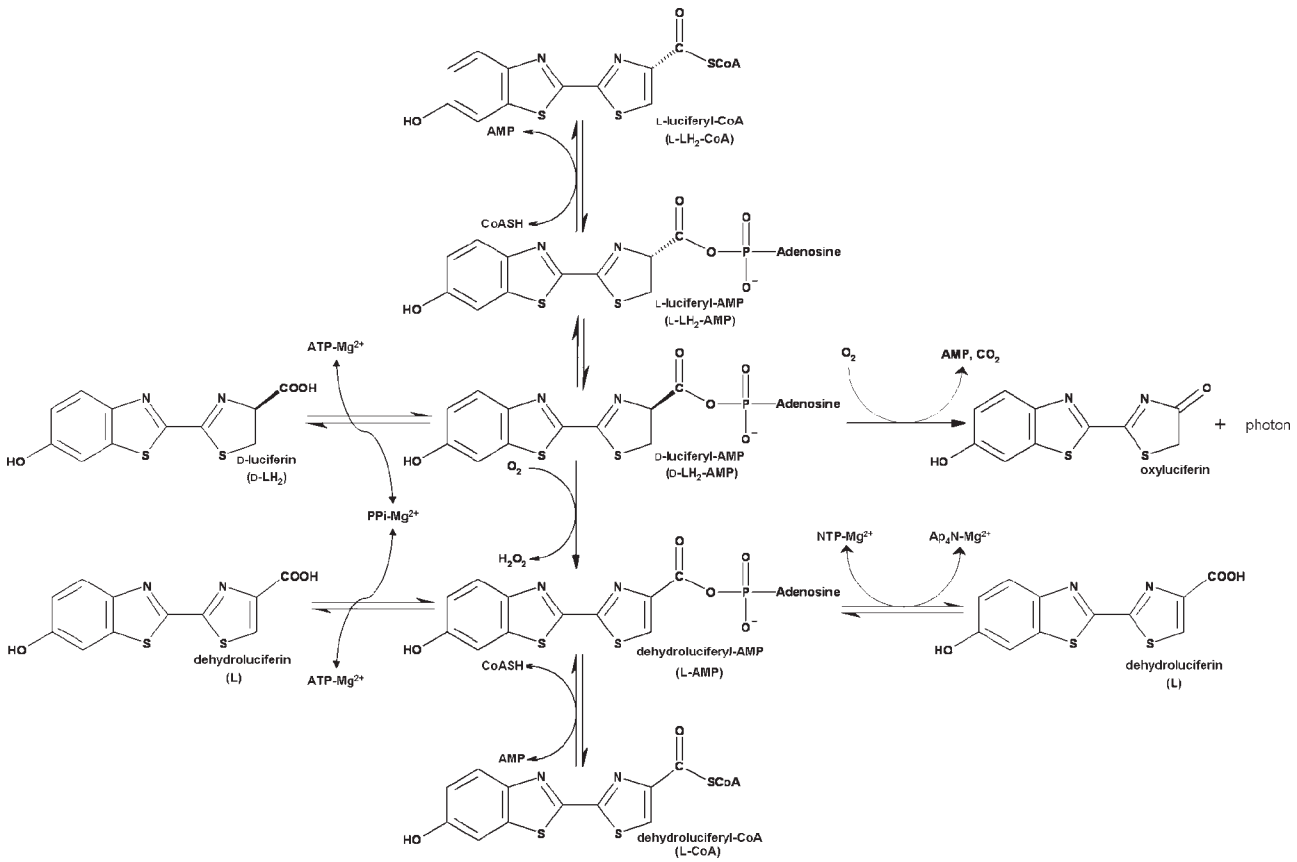

Figure 10. Schematic representation of luciferase catalyzed reactions. The bioluminescent reaction involves the formation of D-LH_2-AMP from D-LH_2 and ATP-Mg^{2+}, with the release of PPi, and its posterior oxidative decarboxylation to oxyluciferin, the light emitter, releasing AMP, CO_2 and a photon. Lateral (dark) reactions includes the oxidation of D-LH_2-AMP to L-AMP, generating hydrogen peroxide (H_2O_2), and its reaction with PPi and/or a nucleoside 5′-triphosphate (NTP), leading to ATP and adenosine (5′)tetraphospho(5′)nucleoside (Ap_4N), respectively, and L. L-AMP and L-LH_2-AMP can react with CoA, generating AMP and the products dehydroluciferyl-coenzyme A (L-CoA) and L-luciferyl-coenzyme A (L-LH_2-CoA). Figure adapted from ref. 24.

FIREFLY LUCIFERIN BIOSYNTHESIS AND THE STEREOSPECIFICITY IN FIREFLY BIOLUMINESCENCE

Although the chemical structure and properties of LH_2 are already established little is known about its biosynthesis (*15*). As already mentioned, the *in vitro* (chemical) synthesis of LH_2 leads to two enantiomers, D- and L-, according to the cysteine isomer used. In Nature only the L-form of amino acids occurs in peptides and proteins, therefore it has been speculated where does the D-cysteine for the synthesis of D-LH_2 comes from.

It was suggested that D-LH_2 could be produced from L-LH_2 by an enzyme-catalyzed inversion of the latter with participation of CoA [Fig. 11; (*56*)]. According to this hypothesis, L-LH_2 is produced from natural L-cysteine, and then L-LH_2 is converted into L-LH_2-CoA which is racemized by enolization (*56*). Hydrolysis of D-LH_2-CoA would give the bioluminescent substrate, D-LH_2. The production of LH_2-CoA is stereospecific (*46*), resulting only from L-LH_2, which indicates that luciferase is able to distinguish the two isomers of LH_2, serving as the acyl-CoA synthetase for L-LH_2, and D-LH_2 being used for the bioluminescent reaction (*46*). This pathway could explain another characteristic of luciferase bioluminescence: the production of light from L-LH_2 (*47*).

For a long time D-LH_2 was regarded as the only isomer capable of producing light (*14, 27*), the L-LH_2 isomer behaving as an inhibitor (*46, 47, 57*), although both D- and L- isomer can be adenylylated by luciferase. Through a mechanism analogous to its synthesis the added L-LH_2 would be adenylylated, converted to L-LH_2-CoA and then racemized and hydrolyzed to D-LH_2. However, the light profile is quite different from the one with directly added D-LH_2, namely presenting a slow increase in the light emission until reaching a plateau, and weaker luminescence intensity (*47*).

Besides the *de novo* synthesis of LH_2 another hypothesis is its regeneration from oxyluciferin, proposed on the basis of the discovery of the luciferin-regenerating enzyme (LRE) in *Photinus pyralis* [Fig. 12; (*58*)]. LRE convertes oxyluciferin to 2-

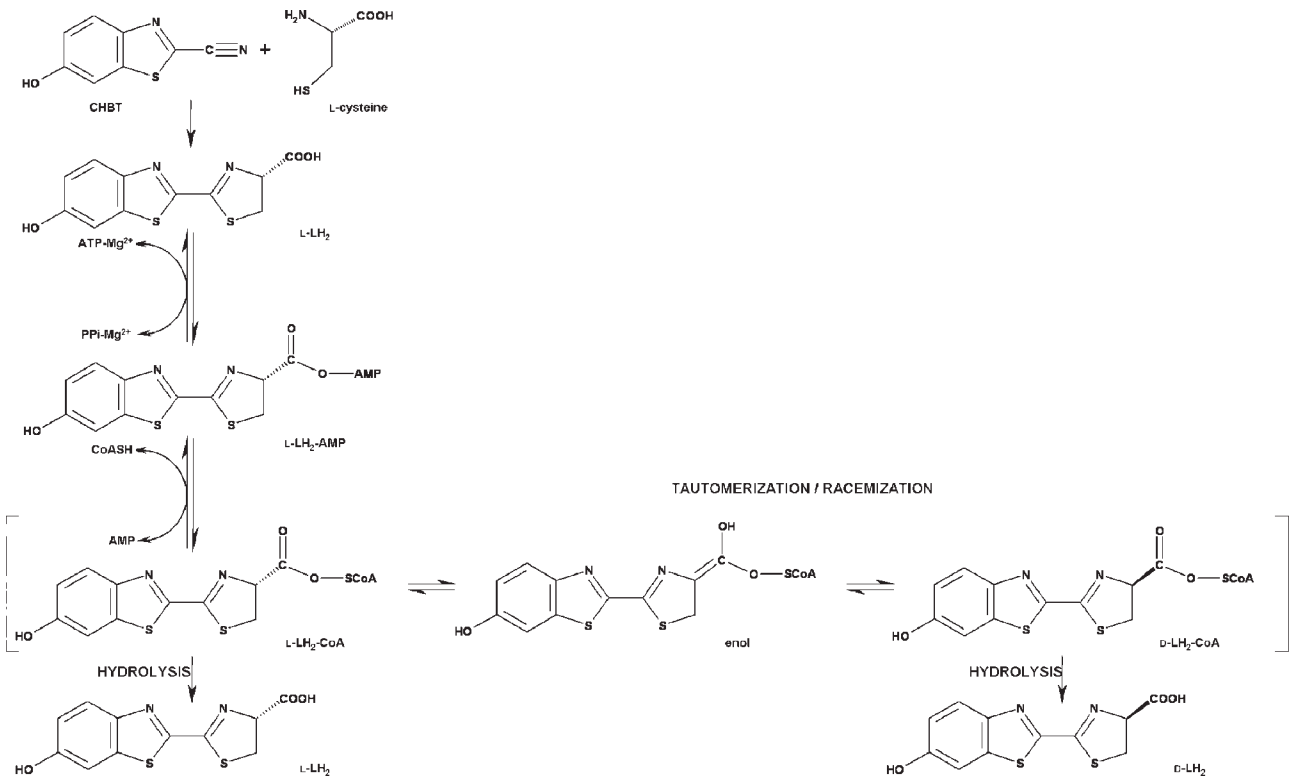

Figure 11. Proposed biosynthetic pathway of D-LH_2 from L-LH_2. Beginning with 2-cyano-6-hydroxybenzothiazole (CHBT) and the natural L-cysteine the L-LH_2 isomer is formed. Through an intermediary step of adenylylation this isomer is converted into L-LH_2-AMP, and its posterior reaction with CoA generates L-LH_2-CoA, being both reactions catalyzed by luciferase. The keto-enol equilibrium leads to racemization of the isomers L- and D-LH_2-CoA which are then hydrolyzed to L-LH_2 and D-LH_2, the latter being the bioluminescent substrate. Figure adapted from ref. 56.

cyano-6-hydroxybenzothiazole (CHBT) and thioglycolic acid. In the presence of D-cystein CHBT was recycled into D-LH_2 by a non-enzymatic reaction. In the same study this activity was detected in extracts from *Luciola cruciata* and *Luciola lateralis*, but further research on this subject is needed (*58*).

APPLICATIONS

The studies aimed to the comprehension and characterization of the bioluminescent reaction allowed the development of luciferase-based techniques, relying in the intrinsic characteristics of enzymatic assays, specificity and sensitivity, and, in the particular case of luciferase, in the possibility of light measurement. In general there are two broad areas of interest: the utilization of *luc* gene as a reporter in molecular biology studies and bioimaging (*59*), or the quantification of analytes connected to ATP or other participant of the light reaction.

The isolation and purification of luciferases from different species was the first step into their practical applications, and today commercial kits are available. The most popular analyte quantified by luciferase is ATP, due to the early discovery that the light intensity is proportional to the ATP concentration in the sample analyzed (*2*) and to the vital importance of this compound to life. On the basis of Eqs. (1) and (2) a simple system can be composed of luciferase, D-LH_2 and the sample to be quantified. Sometimes CoA is also added to the reaction mixture to stabilize the light emitted. ATP quantitation is a fundamental procedure in food industries, as an indicator of bacterial contamination (*60*).

Besides ATP the attention was also turned to other biologically important metabolites which participates in luciferase-catalyzed reaction, namely CoA [through Eq. (5)] (*61*), PPi (*62, 63*) and AMP (*64, 65*).

Recently the discovery that L-LH_2 can also be used by luciferase to produce light led to the development of a new system which involves L-LH_2, ATP, CoA, luciferase and a esterase and was applied to monitoring luciferase concentration itself (*66*). In fact, the use of luciferase to monitor enzyme activity was already applied for pyrophosphatase (PPase) (*67*). The addition of PPi to the assay medium inhibits luciferase, but PPase degrades PPi and thus restores luciferase activity, being the light output proportional to the PPase content (*67*).

Albeit purified luciferase is an important and well established analytical tool it was necessary to wait until recently

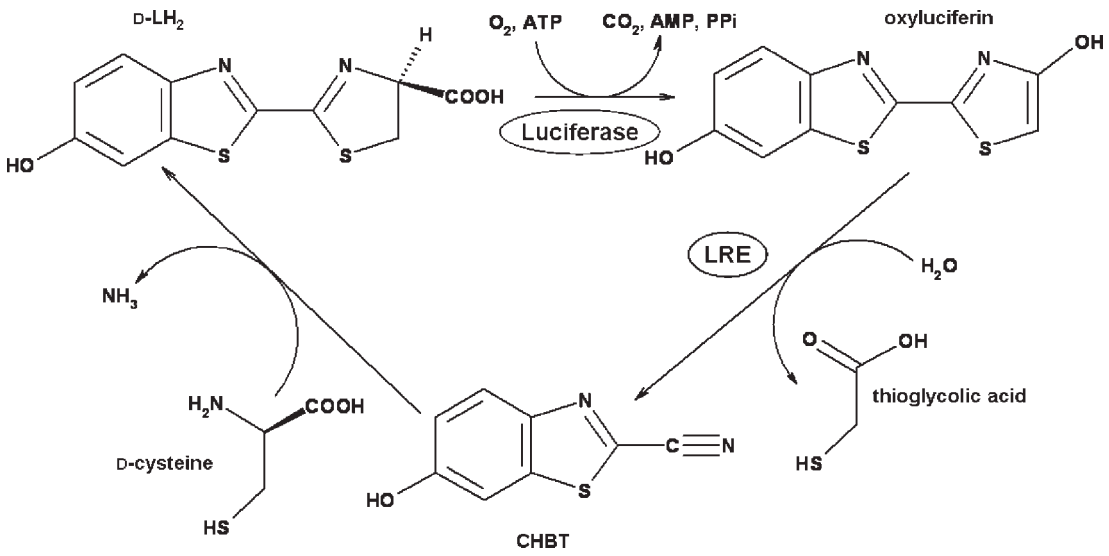

Figure 12. Recycling pathway for LH_2 catalyzed by luciferin-regenerating enzyme (LRE). After the bioluminescent reaction catalyzed by luciferase the natural substrate D-LH_2 is transformed into oxyluciferin. LRE then converts oxyluciferin into 2-cyano-6-hydroxybenzothiazole (CHBT), with the formation of thioglycolic acid as a co-product. In a putative non-catalyzed reaction the condensation of D-cysteine with CHBT produces D-LH_2 that can enter the next cycle of light emission. Figure adapted from ref. 58.

for progresses in molecular biology that allowed the cloning and sequencing of luciferases genes (*68*). But soon after that a new application for luciferases was developed as a reporter gene. A reporter gene is the one which generates a measurable signal according to certain conditions, reporting about cellular functions like the pattern of gene expression, cellular receptors activity, signal transduction pathway, RNA processing and protein–protein interaction (*69*). The *luc* gene is

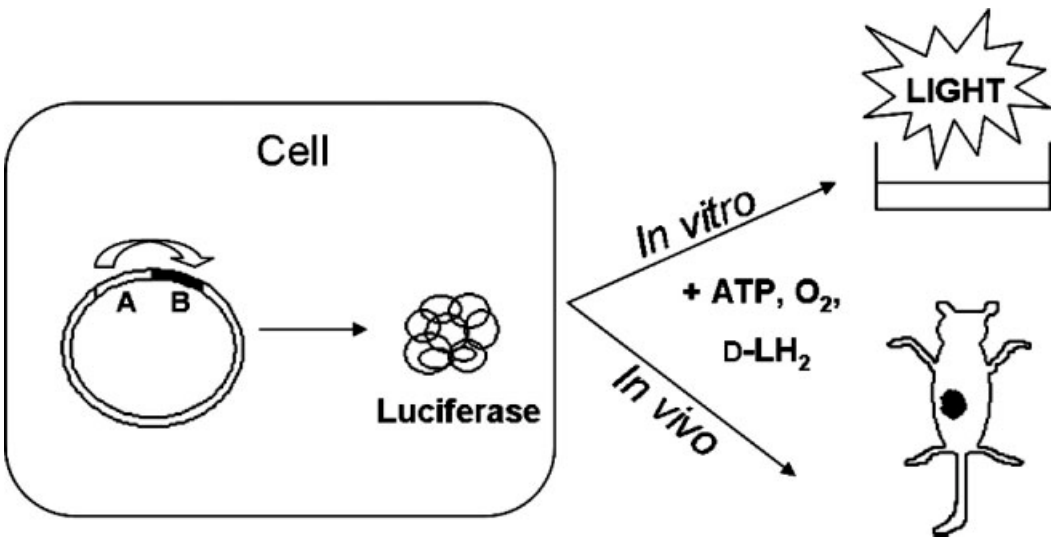

Figure 13. Schematic representation of the reporter gene technology related to luciferase. An expression vector is introduced into the cell. The vector contains a regulatory sequence (A), which will act upon the *luc* gene (B), controlling its expression. When the expression of *luc* is promoted the protein obtained (Luciferase) can be assayed by its light producing activity *in vitro* (cellular extracts) or *in vivo*, placing the animal under a charge-coupled device (CCD) camera that is sensible to detect photons, enabling a luminous spot of light that can be artificially superimposed with a photo of the animal previously taken. In both cases ATP and D-LH_2 must be exogenously administered just before data acquisition. Figure adapted from ref. 59 and text from refs. 59 and 79.

introduced into the desired organism by means of an expression vector, like a plasmid. Inside the cell the *luc* gene is translated into functional luciferase protein. An extract is made and the addition of D-LH_2 and ATP-Mg^{2+} leads to light emission that can be recorded in a luminometer (Fig. 13). This strategy was applied to identify regulatory sequences or promoter regions in the genome (*70, 71*) and in drug screening (*72–74*).

On the basis of this principle another technique was developed: the bioimaging, in which the light production is followed *in vivo* in a whole organism (Fig. 13). This area is receiving much attention and it is applied in important fields like oncology (to evaluate tumor growth and metastases formation) (*75, 76*), infection progression (*74, 77*), and protein expression under specific stimulus (*78*). The principal drawback is that this technique is not yet applied in clinical studies: the actual investigation relies only in animal model (*79*).

Finally, mutagenesis studies on luciferase enabled the production of proteins with new and enhanced properties, like new light color emission (*80*) improved thermostability (*81*), higher luminescence intensity (*82*) and increased catalytic efficiency (*83, 84*).

CONCLUSION

As a complex system that it is, the knowledge about luciferase's bioluminescence is still being built up. Albeit much progress was done during the past decades, many mysteries remain unsolved. In fact even the mechanism of luciferase's action in all reactions shown in this paper is not completely clear until now. Questions as "Which amino acid residues take part in each catalytic step of the reaction?" or "What intermediate enzyme-substrate states are formed during reaction?" are examples of problems that should be elucidated in firefly bioluminescent catalysis. Nonetheless, novel applications based on luciferase are currently being developed and new discoveries are likely to occur in the near future. The unification of so diverse fields as molecular biology, genetics and chemistry will certainly bring to light a new era of bioluminescence research.

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